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Rapid, specific and quantitative assays for the detection of the endophytic bacterium *Methylobacterium mesophilicum* in plants

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Abstract

Xylella fastidiosa is a xylem-limited bacterium that causes citrus variegated chlorosis disease in sweet orange. There is evidence that *X. fastidiosa* interacts with endophytic bacteria present in the xylem of sweet orange, and that these interactions, particularly with *Methylobacterium mesophilicum*, may affect disease progress. However, these interactions cannot be evaluated in detail until efficient methods for detection and enumeration of these bacteria in planta are developed. We have previously developed standard and quantitative PCR-based assays specific for *X. fastidiosa* using the LightCycler® system [Li, W.B., Pria Jr., L.P.M.W.D., X. Qin, and J.S. Hartung, 2003.Presence of *Xylella fastidiosa* in sweet orange fruit and seeds and its transmission to seedlings. Phytopathology 93:953-958.], and now report the development of both standard and quantitative PCR assays for *M. mesophilicum*. The assays are specific for *M. mesophilicum* and do not amplify DNA from other species of *Methylobacterium* or other bacteria commonly associated with citrus or plant tissue. Other bacteria tested included *Curtobacterium flaccumfaciens*, *Pantoea agglomerans*, *Enterobacter cloacae*, *Bacillus* sp., *X. fastidiosa*, *Xanthomonas axonopodis* pv. *citri*, and *Candidatus* Liberibacter asiaticus. We have demonstrated that with these methods we can quantitatively monitor the colonization of xylem by *M. mesophilicum* during the course of disease development in plants artificially inoculated with both bacteria.
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Keywords: *Xylella fastidiosa*; Citrus; Endophyte; Real-time quantitative PCR; LightCycler

1. Introduction

Citrus variegated chlorosis (CVC) is a disease of sweet orange (*Citrus sinensis* L.) trees caused by the xylem-limited bacterium *Xylella fastidiosa* (Chang et al., 1993; Hartung et al., 1994). *X. fastidiosa* causes numerous other diseases of horticulturally important perennial plants in the new world (Hopkins and Purcell, 2002). The pathogen is transmitted by xylem-feeding suctorial insects (sharpshooters) (Lopes, 1999; Almeida and Purcell, 2003) or through seeds (Li et al., 2003). Although *X. fastidiosa* was the first plant pathogen to have its genome completely sequenced (Simpson et al., 2000), there is no effective control for CVC. The pathogen is known to have an extraordinary host range among higher plants in New World ecosystems (Freitag, 1951). Interestingly, within the majority of host plants, *X. fastidiosa* does not damage the host plant (Purcell and Saunders, 1999). In contrast, the horticultural crops that suffer from diseases caused by *X. fastidiosa* have been introduced into New World ecosystems where they become infected with *X. fastidiosa* strains that behave as endophytes in native plants (Chen et al., 2002).

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Endophytic bacteria are those that live in the inner parts of plants without causing apparent damage to their hosts (Hallmann et al., 1997). The role of endophytic bacteria in endophyte–plant associations has been extensively discussed (Araújo et al., 2001, 2002; Hallmann et al., 1997; Lodewyckx et al., 2002). Since endophytes colonize ecological niches similar to those colonized by phytopathogens, interactions between these two groups are possible. Research has shown that some endophytic microorganisms isolated from plant tissues exhibit potential as biocontrol agents against phytopathogens (Sturz et al., 1998) and insects (Azevedo et al., 2000) and also increase plant growth and hasten plant development (Lodewyckx et al., 2002). Possible synergistic interactions between endophytes and phytopathogens that result in increased disease have not yet been described.

Many endophytic bacteria have been isolated from sweet orange (Araújo et al., 2001, 2002) but our research has focused on the genus *Methylobacterium*, which occupies the same ecological niche as *X. fastidiosa* in the xylem vessels of plants (Araújo et al., 2002). Recently, an interaction between *Methylobacterium* spp. and *X. fastidiosa* was strongly indicated (Araújo et al., 2002;Lacava et al., 2004). Lacava et al. (2004) provided evidence that *M. mesophilicum* could reduce the growth of *X. fastidiosa*, while *M. extorquens* could stimulate the growth of *X. fastidiosa* in vitro. The symptoms of CVC could be affected by the populations of *Methylobacterium* spp., other genera of endophytic bacteria and *X. fastidiosa* (Araújo et al., 2002; Koide et al., 2004; Lacava et al., 2004.

In the present study, we developed both standard format and real-time polymerase chain reaction (PCR) assays for detecting *M. mesophilicum*. The methods are based on sequences in the intergenic region of the 16S rDNA region of the genome of this bacterium. The specificity and suitability of the designed primers were tested using standard and real-time PCR in vitro, and also with extracts of *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle) plants doubly inoculated with *M. mesophilicum* and *X. fastidiosa*.

2. Materials and methods

2.1. Bacterial culture and plant inoculations

X. fastidiosa was isolated from sweet orange on solid PW medium (Davis et al., 1981). All strains of endophytic bacteria used in this study were isolated from citrus plants using solid tryptic soy agar amended with benomyl (50 mg/ml) (Araújo et al., 2002) (Table 1). *C. roseus* plants, an excellent experimental host for *X. fastidiosa* (Monteiro et al., 2001), were obtained commercially and maintained in a greenhouse. Inoculations with *X. fastidiosa* and *M. mesophilicum* were done using stem puncture with bacterial cultures at a concentration of ~10^8 CFU/ml (Li et al., 2001). Control inoculations with sterile PW medium were made.

2.2. Design of primers and probes

Primers for the ribosomal operon of Gram-negative bacteria (Table 2) were used to amplify the intergenic

Table 1
Bacterial strains tested with primers MMC1/MMC2

Species (Strain)	Host	Origin
Methylobacterium mesophilicum (SR1.6/6)	Sweet orange *Citrus sinensis*[a]	(Araújo et al., 2002)
Methylobacterium extorquens (AR1.6/2)	*Citrus sinensis*[b]	(Araújo et al., 2002)
Methylobacterium radiotolerans (SR1.6/4)	*Citrus sinensis*[a]	(Araújo et al., 2002)
Methylobacterium zatmanii (SR1.6/2)	*Citrus sinensis*[a]	(Araújo et al., 2002)
Methylobacterium fujisawaense (PR5/4)	Tangerine *Citrus reticulata*	(Araújo et al., 2002)
Curtobacterium flaccumfaciens (ER1/6)	*Citrus sinensis*[c]	(Lacava et al., 2004)
Pantoea agglomerans (ARB18)	*Citrus sinensis*[b]	(Araújo, 2000)
Enterobacter cloacae (PR2/7)	*Citrus reticulata*	(Andreote et al., 2004)
Bacillus sp. (CL16)	Rangpur lime *Citrus limonia*	(Araújo et al., 2001)
Xylella fastidiosa[d] (Fund 3)	*Citrus sinensis*	(Qin et al., 2001)
Xanthomonas axonopodis pv. *citri*[e] (XC62)	*Citrus sinensis*	(Hartung and Civerolo, 1989)
Candidatus Liberibacter asiaticus[f] (B239)	*Citrus sinensis*	J. S. Hartung

[a] Not infected with *X. fastidosa.*
[b] Infected with *X. fastidosa* and showing symptoms of citrus variegated chlorosis.
[c] Infected with *X. fastidosa* but without symptoms of citrus variegated chlorosis.
[d] Causes citrus variegated chlorosis disease. Isolated in São Paulo, Brazil.
[e] Causes citrus canker disease. Isolated in Japan.
[f] Causes citrus greening or huanglongbing disease. Obtained from Taiwan. An extract of an infected plant was used.

Table 2
Sequence of primers used to amplify the intergenic region of the ribosomal operon and to detect *X. fastidiosa* and *M. mesophilicum*

Primer	Target	Sequence 5′-3′	Reference
R1378(−)	16S rRNA genes	CGGTGTGTACAAGGCCC	This work; modified from (Heuer et al., 1999)
F985PTO(−)	16S rRNA genes	AACGCGAAGAACCTTAC	This work; modified from (Heuer et al., 1999)
MMC1	*M. mesophilicum*	TACGTGGAGAGATTCACGGTC	This work
MMC2	*M. mesophilicum*	GTACAAGGCCCGGGAACGTAC	This work
272-1 int	*X. fastidiosa*	CTGCACTTACCCAATGCATCG	(Pooler and Hartung, 1995)
272-2 INT	*X. fastidiosa*	GCCGCTTCGGAGAGCATTCCT	(Pooler and Hartung, 1995)

region of the ribosomal operon of *M. mesophilicum*. We modified the universal primers F985PTO and R1378 (Heuer et al., 1999) by removing 1 and 7 nucleotides, respectively, from the sequences of the 3′ ends of these primers. The PCR product was isolated and purified using the Geneclean Spin Kit (Qbiogene, Carlsbad, CA), cloned into the TOPO TA cloning vector pCR2.1, and the resulting plasmids were introduced into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA). Plasmid DNA was purified using the RPM kit (Qbiogene). DNA sequencing was done at the Biotechnology Center, University of Maryland, College Park, MD. Primer pair MMC1/MMC2 (Table 2) was empirically designed based on this sequence (Fig. 1). The sequence was deposited in GenBank under accession number DQ195080.

2.3. Real-time Quantitative PCR detection assay reaction conditions

The LightCycler® instrument (Roche Diagnostics, Mannheim, Germany) was used to quantify *M. mesophilicum* cells both in vitro and in *C. roseus* seedlings. Hybridization probes (MMCB1: 5′-GCA CTC TAG GGA GAC TG [FL-Q]-3′; and MMCB2: 5′-[LCRed640] GGT GAT AAG CCG CGA GG-[Phop-Q]-3′) were designed based on the sequence of the 390-bp PCR product of primers MMC1 and MMC2 (Fig. 1). Quantitative, real-time PCR reactions were carried out in 18 μl of PCR mixture containing LightCycler® DNA Master Hybprobe mix (*Taq* polymerase, PCR buffer, and dNTPs) (Roche, Indianapolis, IN), 100 nM primers MCC1, 100 nM primer MMC2, 100 nM probe MMC1, 200 nM probe MMC2, and 2.5 mM $MgCl_2$. The amplification program began with 1 cycle of primary denaturation at 95 °C for 30 s followed by 45 cycles of 95 °C for 0 s with a ramp rate of 20 °C/s, 58 °C for 10 s with a ramp rate of 20 °C/s, and 72 °C/s for 20 s with a ramp rate of 20 °C/s. The signal detection setting was F2/F1, and the fluorescence gains were F1=1, F2=15, and F3=30. Known quantities of *M. mesophilicum* strain SR1.6/6 were used as templates for real-time PCR and for viable cell counts.

2.4. Standard PCR assay conditions

Standard PCR assays were performed with primers 272-1-int and 272-2-int specific for *X. fastidiosa* (Pooler and Hartung, 1995) and primers MMC1 and MMC2

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5′AACGCGAAGAACCTTACCATCCTTTGACATGGCGTGTTACGTGGAGAGATTCACGGTCCACTTCGGTGGCGCGCACAC
3′TTGCGCTTCTTGGAATGGTAGGAAACTGTACCCGACAATGCACCTCTCTAAGTGCCAGGTGAAGCCACCGCGCGTGTG

5′AGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAGCGAGCGCAACCCACGTCCTTA
3′TCCACGACGTACCGACAGCAGTCGAGCACAGCACTCTACAACCCAATTCAGGGCGTCGCTCGCGTTGGGTGCAGGAAT

5′GTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCGGTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCC
3′CAACGGTAGTAAGTCAACCCGTGAGATCCCTCTGACGGCCACTATTCGGCGCTCCTTCCACACCTCCTGCACTTCAGG

5′TCATGGCCCTTACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAGGCGAAGGAGCGATCTGGAGCAA
3′AGTACCGGGAATGCCCTACCCGATGTGTGCACGATGTTACCGCCACTGTCACCCTCCGCTTCCTCGCTAGACCTCGTT

5′ATCCCCAAAAGCCGTCTCAGTTCGGATTGCACTCCGCAACTCGAGTGCATGAAGGCGGAATCGCTAGTAATCGTGATC
3′TAGGGGTTTTCGGCAGAGTCAAGCCTAACGTGAGGCGTTGAGCTCACGTACTTCCGCCTTAGCGATCATTAGCACTAG

5′AGCATGCCGCGGTGAGTACGTTCCCGGGCCTTGTACACACCG
3′TCGTACGGCGCCACTCATGCAAGGGCCCGGAACATGTGTGGC
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Fig. 1. Amplicon from the intergenic region of the rDNA of *M. mesophilicum* strain SR1.6/6 used to develop the PCR assays. The primers for universal amplification and sequencing of the region are shown in *italics*. The sequences of the specific amplification primers MMC1 and MMC2 are shown in **bold**. Sequences of the LightCycler® hybridization probes are underlined.

specific for *M. mesophilicum* (Table 2) in a final reaction volume of 40 µl. The amplification conditions used were one cycle of 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1.5 min, with a final extension cycle of 10 min at 72 °C. PCR products were visualized by staining with ethidium bromide following electrophoresis through agarose gels. The expected amplification products were 472 and 390 bp for *X. fastidiosa* (Pooler and Hartung, 1995) and *M. mesophilicum* (Fig. 1), respectively. DNA was extracted from *C. roseus* petioles as described previously (Li et al., 2003).

3. Results

3.1. Specificity and sensitivity of primers

The specificity of primers MMC1 and MMC2 for *M. mesophilicum* was tested by performing the PCR with bacterial DNA isolated from cultured *M. mesophilicum*, *M. extorquens*, *M. radiotolerans*, *M. zatmanii*, *M. fujisawaense*, *Curtobacterium flaccumfaciens*, *Pantoea agglomerans*, *Enterobacter cloacae*, *Bacillus* sp., *X. fastidiosa*, and *Xanthomonas axonopodis* pv. *citri* (Table 1). Extracts containing *Candidatus* Liberibacter asiaticus (Jagoueix et al., 1997), the causal agent of citrus greening or huanglongbing disease, were prepared from greenhouse grown infected plants. The expected amplification product was detected only in extracts of *M. mesophilicum* (Fig. 2). The sensitivity of this set of primers was estimated in standard PCR using serial dilutions of cultured bacteria and amplicons were observed through the 10^{-6} dilution, which contained 130 viable cells in the amplification reaction (Fig. 3).

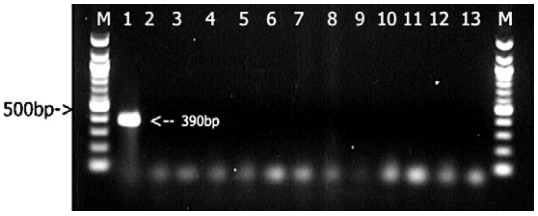

Fig. 2. The specificity of the primers MMC1 and MMC2 for *M. mesophilicum* in the PCR with various strains of endophytic or pathogenic bacteria isolated from citrus. M, 100 bp DNA ladder; Lane 1, *M. mesophilicum* (SR1.6/6), Lane 2, *M. extorquens*, Lane 3, *M. radiotolerans*, Lane 4, *M. zatamani*, Lane 5, *M. fujisawaense*, Lane 6, *C. flaccumfaciens*, Lane 7, *P. agglomerans*, Lane 8, *E. cloacae*, Lane 9, *Bacillus* sp., Lane 10, *X. fastidiosa* (cvc), Lane 11, *X. axonopodis* pv. *citri* (citrus canker), Lane 12, *Candidatus Liberibacter asiaticus* (citrus greening, assayed as a plant extract), Lane 13, negative control (water).

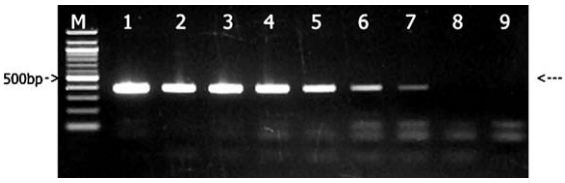

Fig. 3. Sensitivity of the PCR with the MMC1/MMC2 primer set for the detection of *M. mesophilicum*. M, 100 bp DNA ladder; Lanes 1–8, single colony serially diluted 10-fold. Lane 9 negative control (water). The PCR product, indicated by the arrow, is 390 bp.

3.2. Detection of *M. mesophilicum* and *X. fastidiosa* in *C. roseus* by standard PCR

Twenty days after inoculation of *C. roseus* seedlings, *M. mesophilicum* was detected by PCR using primers MMC1 and MMC2 (Fig. 4). *X. fastidiosa* was also specifically detected using primers 271-int and 272-int on extracts of *C. roseus* inoculated with *X. fastidiosa*, but not in seedlings simultaneously inoculated with both *X. fastidiosa* and *M. mesophilicum*. However, in such plants inoculated with both bacteria, *M. mesophilicum* was detected by PCR with primers MMC1 and MMC2 (Fig. 4).

3.3. Detection and quantification of *M. mesophilicum* in *C. roseus* seedlings by quantitative, real-time PCR

We established a standard curve relating turbidity to colony forming units for cultured *M. mesophilicum* by

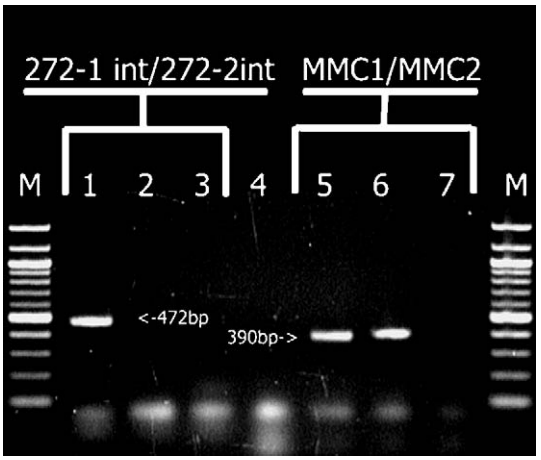

Fig. 4. Detection of *M. mesophilicum* and *X. fastidiosa* in artificially inoculated *C. roseus* by PCR with the MMC1/MMC2 and 272-1 int/272-2 int primer sets. M, 100 bp DNA ladder; Lane 1, DNA extract from petioles inoculated with *X. fastidiosa*; Lane 2, DNA extract from petioles inoculated with *X. fastidiosa* and *M. mesophilicum*; Lane 3, PW media culture; Lane 4, negative control (water); Lane 5, DNA extract from petioles inoculated with *X. fastidiosa* and *M. mesophilicum*; Lane 6, DNA extract from petioles inoculated with *M. mesophilicum*; Lane 7, PW media culture.

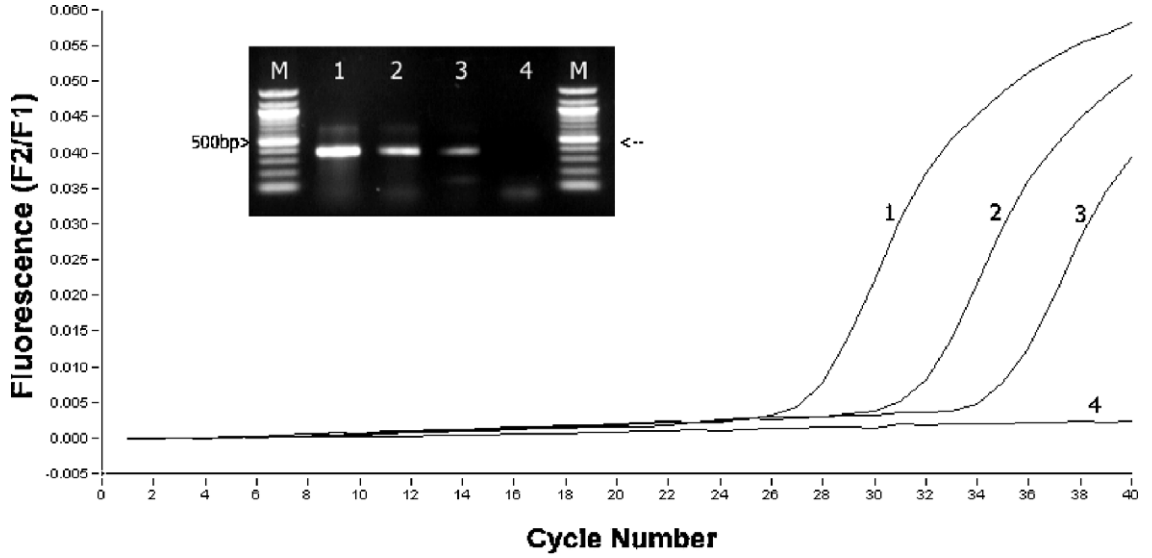

Fig. 5. Quantitative, real-time amplification plot of *M. mesophilicum* target. Curves 1 to 3 are from a dilution series of strain SR1.6/6. The reactions contained 9×10^5 to 9×10^3 cells, respectively; curve 4, water only. Inset: agarose-gel analyses of the end-point PCR results. M, 100 bp DNA ladder. The PCR product, indicated by the arrow, is 390 bp.

dilution plating, and aliquots from the same dilution series were used in the LightCycler® assay to establish a standard curve relating C_t to genome equivalents in the bacterial extracts (Fig. 5). DNA was extracted from *C. roseus* petioles collected 44 days after inoculation with bacteria either singly or in combination, and was used in the Lightcycler reactions to estimate the populations of *X. fastidiosa* and *M. mesophilicum* present in

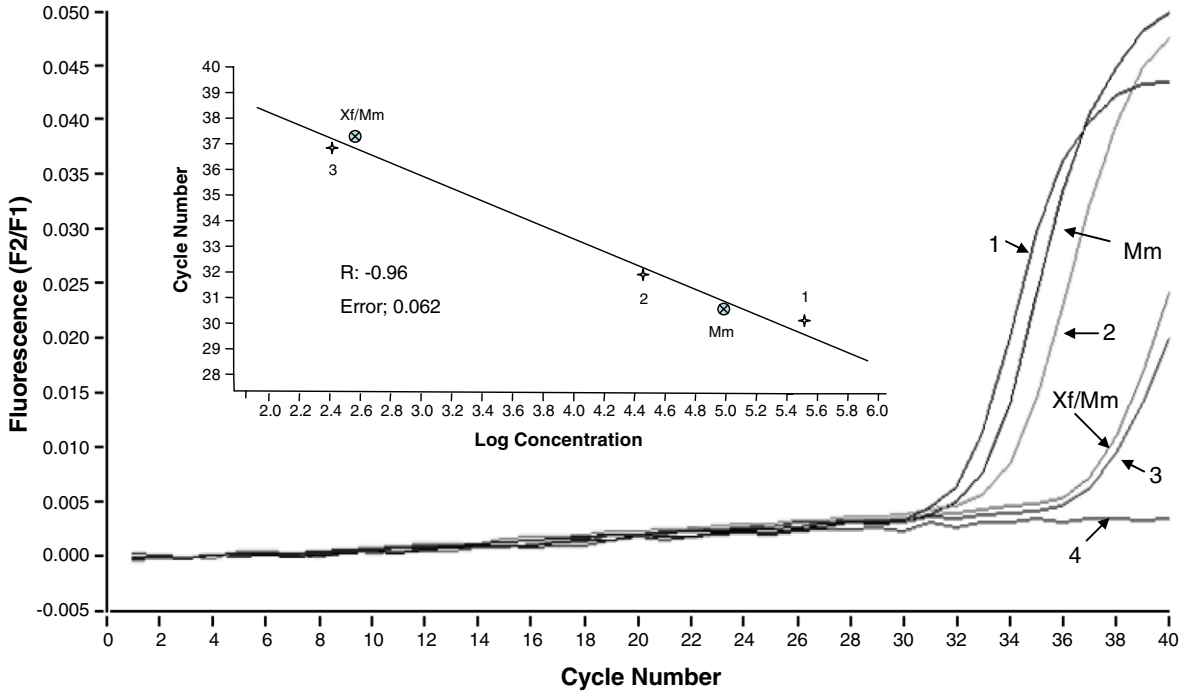

Fig. 6. Quantitative, real-time amplification plot of *M. mesophilicum* (Mm) targets. Curves 1 to 3 are from a dilution series of strain SR1.6/6 of *M. mesophilicum*; the reactions contained 3.5×10^5 to 3.5×10^2 cells respectively and curve 4, water only. The curve labeled Mm is from a DNA extract of *C. roseus* petioles from plants inoculated with *M. mesophilicum* only. The curve labeled Xf/Mm is from a DNA extract of *C. roseus* petioles from plants doubly inoculated with *M. mesophilicum* and *X. fastidiosa* (Xf).

the extracts. The number of *M. mesophilicum* cells detected in plants inoculated with *M. mesophilicum* alone was 4.2×10^4 and the number of *M. mesophilicum* cells detected in plants inoculated with both *M. mesophilicum* and *X. fastidiosa* was only 3.9×10^2, a 100-fold reduction (Fig. 6).

4. Discussion

DNA-based methods, such as the conventional PCR, have been increasingly used for rapid and sensitive analyses of samples containing bacteria. This sort of approach is especially useful for detection and quantification of slow growing and difficult to culture bacteria such as *X. fastidiosa* and *M. mesophilicum*. This article describes the development of specific primers and probes for detection and quantification of the endophytic bacterium *M. mesophilicum* in plant tissues by both standard format PCR and real-time PCR assays. This is the first report of a quantitative PCR detection method for endophytic bacteria from sweet orange, as well as the first report of quantification of *M. mesophilicum* using the LightCycler® hybridization probe assay. The assays will provide a useful complement to current assays for *X. fastidiosa* that utilize the same formats (Li et al., 2003), and so can be conveniently run on the same sets of samples.

The primer pair MMC1 and MMC2 was designed based on comparison of the newly determined sequence of the intergenic region of the ribosomal operon of strain SR1.6/6 to that of related bacteria in GenBank. The specificity of primers MMC1/MMC2 for *M. mesophilicum* was confirmed in the laboratory with DNA samples from endophytic bacteria frequently isolated from citrus (Araújo et al., 2002) as well as from pathogens that cause important citrus diseases including *X. fastidiosa* (citrus variegated chlorosis) (Hartung et al., 1994), *X. axonopodis* pv. *citri* (citrus canker) (Graham et al., 2004) and *Candidatus Liberibacter asiaticus* (citrus greening or huanglongbing) (Jagoueix et al., 1997).

The endophytic bacterium *M. mesophilicum* was detected in extracts of *C. roseus* 20 days after inoculation using primer pair MMC1/MMC2 in a standard PCR assay. In a similar experiment, where both *M. mesophilicum* and *X. fastidiosa* were inoculated into *C. roseus*, it was possible to detect both the endophyte and the pathogen by standard PCR. These data demonstrate that primer pair MMC1/MMC2 specifically detects *M. mesophilicum* (strain SR1.6/6) in plant tissue in the presence of other biologically significant bacteria.

Li et al. (2003) used the LightCycler® system to detect *X. fastidiosa* in fruits and seeds of sweet orange. These authors plated *X. fastidiosa* onto culture media to establish the standard curve relating fluorescence to viable cells in the quantitative PCR assay. In the present study, we used the same method to establish the standard curve with *M. mesophilicum*. Our successful detection and quantification of *M. mesophilicum* cells in petioles of *C. roseus* confirmed that it is possible to use the LightCycler® to study the interaction between this endophytic bacterium and *X. fastidiosa*.

As an example, the co-inoculation of *C. roseus* with *M. mesophilicum* and *X. fastidiosa* demonstrated that the population of this endophytic bacterium was lower in the presence of *X. fastidosa* than when it was allowed to colonize *C. roseus* without *X. fastidiosa* and that the population of *X. fastidiosa* was in turn reduced by *M. mesophilicum*. These initial results from a single time point only, are consistent with other data that suggest interactions between *M. mesophilicum* and *X. fastidiosa* in vitro (Lacava et al., 2004). This method for the detection and enumeration of *M. mesophilicum* in planta, combined with our previously described method for detection and enumeration of *X. fastidiosa* in planta (Li et al., 2003), provide tools to quantify the interaction between *M. mesophilicum* and *X. fastidiosa*. In future experiments, these methods will be used to study the dynamic interactions of these bacteria in orchard grown citrus infected with both bacteria.

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